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Newer Developments in HPLC Impacting Pharmaceutical Analysis: A Brief Review

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1. INTRODUCTION

High-performanceliquid chromatography (HPLC) is the premier analytical technique used in many pharmaceutical applications including potency/purity/performance assays, pharmacokinetics/ bioanalytical testing, purification, high throughput screening (HTS),

In Process Control (IPC) Monitoring and Quality Control (QC) testing [1-6]. The pharmaceutical industry is the major consumer segment of HPLC and has been the primary driving force for higher throughput and performance. This article provides a brief review of significant developments in HPLC impacting pharmaceutical analysis in the last decade. Instrumentation: Ultra-high pressure LC (UHPLC) going mainstream. Columns: Sub-2 μm, sub-3μm core-shell and hybrid particles; novel bonding chemistries; hydrophilic interaction chromatography (HILIC); immobilized polysaccharide chiral phases; columns for biomolecules and biopharmaceuticals. Others: Liquid chromatography – Mass Spectrometry (LC/ MS) - particularly High-Resolution MS (HRMS) or Hybrid MS; Charged Aerosol Detector (CAD); Automated Method Development Systems (AMDS).

yet comprehensive update of important HPLC developments, with each topic supported by brief descriptions of practical benefits/applications, critical commentaries from a user's perspective, and key references.

Ultra-high-pressuree Liquid Chromatography (UHPLC):

The "revolution" in ultra-high pressure LC (UHPLC) began in 1997 with the proof-of-concept study by Professor James Jorgenson , followed by the first commercial system introduced in 2004 . Today, the transformation from HPLC to UHPLC is mostly complete with all major manufacturers having some type of

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UHPLC offerings. Detailed reviews of UHPLC systems, columns and applications are available elsewhere . Fundamentals, benefits, potential issues and best practices of UHPLC in pharmaceutical analysis are well documented.

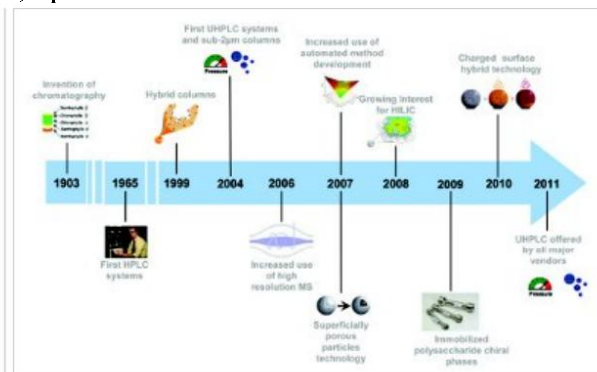

Figure 1. Newer developments (selected) in HPLC impacting pharmaceutical analysis in the last decade.

Note that higher system pressures allow the use of columns packed with smaller particles (e.g., sub-2 µm) for faster analyses (Figure 2) or superior separations of complex samples (Figure 3). All UHPLC have low system dispersion (~10 to 20 µL for 4σ bandwidths) from the use of improved injectors, smaller ID tubing (< 0.005”) and smaller UV detector flow cells (0.5 – 2 µL) . The use of 2.1 - 3.0 mm ID columns packed with sub-2 µm or sub-3 µm particles is typical. Other important system characteristics include smaller system dwell volumes (0.1 – 0.3 mL) and faster detector response/data acquisition (>40 pt/s) for highthroughput applications. The benefit of faster analysis of UHPLC vs. conventional HPLC is illustrated in Figure 2 in a method transfer case study for a drug product. Using a geometrical scaling of column and operating parameters from HPLC to UHPLC , a reduction of analysis time up to tenfold with similar resolution, is not unusual. This benefit of “faster analysis with good resolution” provides the primary incentive for most users to consider the purchase of the more expensive UHPLC equipment.

Another important benefit of UHPLC is its superior separation of complex samples. This aspect is often overlooked and appears to be “underreported” in the literature . Peak capacities (PC) in the range of 400 to 1000 in a reasonable time span (~1h) have been demonstrated using UHPLC. Peak capacity is the number of peaks that can be resolved in the chromatogram with a resolution of 1.0; typically ~200 for conventional HPLC .

For the first time ever, UHPLC can offer more effective assays, in a single dimension, for complex pharmaceuticals, natural materials, and other difficult sample matrices . Figure 3a and 3b show two applications (a plant extract and a protein tryptic digest) to illustrate the current capability of UHPLC in highresolution separations with Pc >300. Other benefits of UHPLC include substantial solvent savings (5-15 fold), increased mass sensitivity (3 – 10 fold) and precision performance for both retention times (2 - 3 fold) and peak areas

System characteristics of UHPLC	Range and Comment
High pressure limit	15,000 to 19,000 psi (1000 to 1250 bar) with flow rate limits of 2 to 5 mL/min. Compatible with conventional and sub-2 µm particle columns.
Low system dispersion	Instrumental bandwidth of 5 to 20 µL (4 σ) depending on configuration. System band broadening reduced by using smaller connection tubing <0.005" I.D. and small UV flow cells (0.5 to 2 µL). Compatible with columns down to 2 to 3 mm ID.
Low gradient dwell volume	100 to 400 µL (higher for quaternary pumps). Compatible to high throughput screening (HTS). Small dwell (mixing) volumes may negatively impact UV detector noise.
Others	Fast injection cycle (~20s) and detector response, and high acquisition rate (>40 pt/s) for HTS. Compatibility to existing HPLC methods desirable (e.g., flow range, column oven size, sample loop).
Benefits of UHPLC	**Comment**
High throughput	Increase throughput by 3 -10 fold vs. conventional HPLC while maintaining similar resolution, e.g., 5 min vs. 20 min purity analysis.
Rapid method development	Fast analysis with short columns is ideal for rapid column and mobile phase screening and method optimization.
High resolution	Increase resolution by up to 3 fold vs. HPLC, e.g., Peak capacities (P_c) 400 – 600 vs. ~200 for HPLC.
Solvent saving	Typical 5 to 15 fold reduction vs. HPLC due to shorter analysis time and use of smaller ID columns.
Higher sensitivity	3 to 10 fold increase of mass sensitivity (reduction of sample amounts injected). Use of long-pathlength UV flow cells (50 – 60 mm) can increase concentration sensitivity up to six times
Higher precision	Significant increase of retention time (2 - 3 fold) and peak area precision (<0.1% RSD achievable at injection volumes >1 µL)
Can be combined with other approaches	UHPLC is compatible with high-temperature LC, 2D-LC, or core shell columns individually or in combination. These are optional rather than alternative approaches.

HPLC COLUMN AND STATIONARY PHASE DEVELOPMENTS:

HPLC column is the heart of the chromatographic system. The

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pharmaceutical industry has been the primary driver for HPLC columns towards higher speed, resolution, and better peak shapes for basic analytes. In addition, QC laboratories have demanded improved column batch-tobatch reproducibility. From the 1970s to 1990s, there were steady quality improvements of the packing materials accompanied by a gradual reduction of "standard" particle sizes from 10 to 3 µm . The introduction of high-purity type B silica materials (with low metallic content) in the late 1980s was a huge step and resulted in reduced silanol activity and substantial improvements in lot-to-lot consistency . The use of high-purity silica is now the norm for all modern silica-based columns.decreasing in the past five decades cumulating to the use of sub-2 µm microparticulate silica in the early 2000s. As predicted, these particles (e.g., 1.7 µm) yield excellent efficiency performance (~280,000 plates/m or plate height of ~4 µm). However, columns packed with sub-2 µm particles generate high back pressures and are typically packed in a 2.1-mm ID format to reduce efficiency loss from viscous heating effects . The system requirements for high pressures and low dispersion (to reduce extracolumn band broadening) were responsible for the current characteristics of modern UHPLC systems. Further reduction of particle size to less than 1.5 µm may be advantageous for even higher speed and performance. However, it must be accompanied by a substantial increase of system pressures and a reduction of column ID to capillary formats.

CORE-SHELL PARTICLES

The concept of fused-core or core-shell particles for reducing resistance to mass transfer was first described by Kirkland . The first core-shell particles had these characteristics: 2.7 µm superficially porous silica materials with nonporous cores (1.7 µm) and porous shells (0.5 µm thick) . These sub-3 µm particles appear to have similar efficiencies as the sub-2 µm fully porous materials but generate much lower pressure drops. The exceptional performance may be due to their shorter diffusion paths of the shells and/or the narrower distribution of the packings. Core-shell columns are rapidly gaining wide acceptance for fast separations (HTS, IPC and cleaning verification) and for biomolecules . Myriad bonded phases and particle sizes (1.3, 1.7, 2.6, 2.7 and 5 µm) are currently available from an increasing number of manufacturers (6+). We fully expect these columns to be highly competitive in all applications to those from porous microparticulates .

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Hydrophilic Interaction Chromatography (HILIC):

The retention of many highly polar compounds is simply unachievable or problematic due to phase collapse (bonded phase “dewetting”) under reversed-phase LC (RPLC) conditions in mobile phases with low organic contents . The HILIC mode, first developed by Alpert in 1990s , uses a hydrophilic stationary phase (silica, diol, cyano, amide, ztwitterionic) with an RPLC-like aqueous buffer and acetonitrile mobile phases, has enjoyed increasing popularity for the analysis of polar drugs, secondary drug metabolites, amino acids, peptides, neurotransmitters, oligosaccharides, carbohydrates, nucleotides, or nucleosides . The actual retention mechanism in HILIC can be the “partitioning” of analyte molecules to the water layer adhering to the hydrophilic bonded groups. Other prominent benefits of HILIC include “orthogonal” selectivity to RPLC where sample preparations are compatible to both modes, higher electrospray ionization sensitivity for MS (5-15 fold), and lower operating pressures vs. RPLC.

improved versions of the highly successful coated polysaccharides chiral stationary phases (CSPs) were available in the late 2000s. They brought similar versatility as the earlier coated CSPs but are more robust to aggressive solvents and can be used in normal phase, polarorganic and reversed-phase modes.

Wide-pore silica and polymeric packings first available in 1980s were effective for separations of large biomolecules . With the advent of recombinant proteins as biopharmaceuticals such as monoclonal antibodies (mAb), the need for detailed characterization by HPLC or capillary electrophoresis for QC purposes has become more pressing . Recent developments of sub-2 μmmicroparticulates and coreshell wide-pore particles as well as several innovative ion-exchange and size exclusion phases have proved useful for separations of these large biologics.

The combination of HPLC with mass spectrometry (LC/MS) has been touted as the perfect analytical tool, combining the separation power of HPLC and the unsurpassed sensitivity/selectivity of MS. LC/MS is the preferred technique for identification of impurities and degradants,HTS in drug discovery, bioanalytical assays (LC/MS/MS for drugs and metabolites in biofluids), and in-progress monitoring during process scale-up for the synthesis of drug substances .

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LC/MS is becoming the standard platform technology for cleaning verification of highly-potent drugs and determination of potential genotoxic impurities . The last decade has seen the rapid evolution of HRMS (such as time-of-flight (TOF), OrbiTrap MS) and hybrid MS (such as Quadrupole-TOF or ion trap-OrbiTrap). The combination of HRMS with UHPLC and 2-D LC has enabled active research in metabonomics, proteomics, de nova protein sequencing, and characterization of biopharmaceuticals . Perhaps the most exciting opportunity for LC/MS lies ahead as a generic platform technology for the determination of disease biomarkers and clinical diagnostics in the emergent fi eld of personalized medicine.

The lack of an ideal universal detector is often cited as a limitation of HPLC, though the UV/Vis detector comes fairly close for chromophoric compounds. The refractive index detector is not gradient compatible and does not have sufficient sensitivity . Evaporative light scattering detection (ELSD) using nebulizer technology with laser light scattering detection is an option and is gradient compatible but has been recently surpassed by CAD (uses nebulizer with corona discharge detection), which offers better sensitivity (low ng) and improved linearity. CAD is becoming a mainstream detector for HTS in medicinal chemistry, reaction monitoring, and raw material/excipient testing.

HPLC method development for complex mixtures is time-consuming due to the need to optimize many operating parameters (column dimension; type of bonded phase and mobile phase A and B (organic solvent/buffer type, pH, and ionic strength), gradient time and range, column temperature, and flow rate) . A common example is the stability-indicating or purity assay of active pharmaceutical ingredients (API) in which all impurities and degradants must be separated for accurate quantitation by UV detection. Software or automated systems based on simulation, prediction, simplex optimization, and column/mobile phase screening have been available to facilitate HPLC method development for many years. Continued improvements have enhanced their capability and ease-of-use though they never appeared to be very popular . The latest market entry was an add-on software package compatible to two of the commonly used chromatography data systems. The software addresses the most time-consuming portion of the HPLC method development process (optimization) by automating method sequence from a user-defined design space using principles of Design of Experiments (DoE) and

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Quality by Design (QbD) . This software can also perform statistical analysis and display optimum conditions after importing of the completed sequence results. Figure 5 illustrates the concepts and salient features of this software as implemented in our laboratory. Since concepts of DoE and QbD are well accepted, initial customer interest appeared to be high. AMDS is particularly useful to laboratories specializing in method development to support earlyphase drug development . For instance, to support process developments for API synthesis and drug product manufacturing from complex drug molecules with multiple chiral centers, as many as 10-20 HPLC methods (achiral and chiral methods for raw materials, starting materials, intermediates, final drug substances and drug products) are typically developed in quick succession.

2. CONCLUSION

In summary, HPLC remains a highly dynamic field with numerous innovations in instruments, column technologies, and approaches in recent years. Pharmaceutical scientists are early adopters and beneficiaries of these newer technologies for research, development and quality control. UHPLC is becoming the standard HPLC platform with rapid adoption by research & development, albeit slower implementation in QC labs. Newer column technologies allow faster and more efficient analysis of complex samples, chiral molecules and biomolecules. Finally, the rapid advancements of UHPLC and 2-D LC in combination with high-resolution MS have revolutionized life science research and promise to have even greater impact for clinical diagnostics. These developments are welcomed progress for the analytical chemist working in this rapidevolving world of drug development.

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